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Ubiquitination of endogenous calmodulin in rabbit tissue extracts

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Previously we were able to show that *purified* calmodulins from vertebrates, plants (spinach) and the mold *Neurospora crassa* can be covalently conjugated to ubiquitin in a Ca^{2+}-dependent manner. It was therefore pertinent to answer the question if a tissue extract contains all the components necessary for the endogenous synthesis of ubiquityl calmodulin (uCaM). Therefore [^{125}I]ubiquitin, ATP/Mg^{2+} and Ca^{2+} were added to tissue extracts enriched by a single ion exchange step. In such extracts of red blood cells, skeletel muscle and testis a novel ubiquitin conjugate of 27–29 kDa is formed. This novel band could be identified as ubiquityl-calmodulin by the following methods: (i) identical R_f-value of novel conjugate and standard uCaM in SDS-PAGE; (ii) Ca^{2+}-dependent conjugate formation; (iii) Ca^{2+}-dependent adsorption to fluphenazine-Sepharose; (iv) Ca^{2+}-dependent mobility change of the novel conjugate during SDS-PAGE; and (v) inhibition of conjugate band formation by phosphorylase kinase. These experiments clearly demonstrate that ubiquityl calmodulin can be endogenously generated in enriched cellular extracts and strongly indicate that this reaction is of importance in vivo.

Calmodulin; Ubiquitin; Calmodulin–ubiquitin conjugate; Protein-ubiquitination; ATP-dependent proteolysis; Trimethyl lysine

1. INTRODUCTION

Ubiquitin, a heat stable polypeptide of 8.5 kDa, has been shown to be involved in covalent modification reactions in eukaryotic cells (for reviews see [1,2]). Proteins which have been detected as modified in vivo include histone (uH2A) [3], actin (arthrin) [4], phytochrome [5] and receptors [6,7]. Although the detection and isolation of these conjugates from tissues indicates a high significance of the protein modifications for the cell, much is still to be learned on their synthesis and biological function.

Previously [8–12] we have shown that vertebrate, plant and mold calmodulins can be covalently coupled to ubiquitin in the presence of Ca^{2+} and ATP/Mg^{2+} by ubiquityl-calmodulin synthetase (uCaM-synthetase) from mammalian tissues, for which a specific affinity-based assay has been described [9]. The calmodulins are multiply ubiquitinated at a single site leading to a poly-ubiquitin tail [11,12]. Evidence has been presented that only the free form of calmodulin can be modified [9].

However, all of these findings have been made with purified isolated calmodulins under in vitro conditions and until now it has not been possible to detect ubiquitin conjugates of calmodulin in cellular extracts or intact cells. A major open question therefore is whether the ubiquitination of calmodulin is a reaction which normally occurs in viable cells and if so whether it can be detected in cell-free extracts or enriched fractions (see also preliminary report in [13]), which contain a protein composition similar to that of the cytosol.

In this paper it will be shown that simply the addition of radioactively labelled [^{125}I]ubiquitin, ATP/Mg^{2+} and Ca^{2+} to cellular extracts enriched by a single ion-exchange step lead to the production of ubiquitinated endogenous calmodulin. This makes it very probable that the ubiquitination of calmodulin belongs to the normal regulatory repertoire of eukaryotic cells.

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2. MATERIALS AND METHODS

Enriched rabbit tissue/cell extracts from reticulocytes (APF II, ATP-dependent proteolysis fraction II), erythrocytes, skeletal muscle (white, fast twitch; red, slow twitch) and testis were prepared by a single chromatographic step on DEAE-Sephacel (Pharmacia, Uppsala) as previously described in [13,14]. The DEAE-cellulose method employed for the enrichment of the tissue extracts is based on the method of preparing the ATP-dependent proteolytic fraction II [10,14,15] also called APF II. In this step proteins like hemoglobin, myoglobin and especially ubiquitin (see [1,5]) run unadsorbed through the column. The ubiquitin conjugating activity including uCaM-synthetase [14], calmodulin and many other proteins are adsorbed, enriched and then desorbed by increasing the NaCl concentration to 350 mM [10,14]. Ubiquitin was prepared according to [14] or purchased from Sigma (Munich). The ubiquitin concentration necessary for half-maximal activation of ATP-dependent proteolysis [14] as tested with reticulocyte APF II [14] was 1.5–2 μM. ^{125}I-labelled ubiquitin was synthesized according to the chloramine-T procedure (i.e. ^{125}I-CT-ubiquitin, 0.1–6×10^8 cpm/mg) [14,16]. Bovine testis calmodulin was isolated according to [17] and purified further by affinity chromatography according to [18]. The biological activity of the purified calmodulin was tested [19] with phosphorylase kinase in an AutoAnalyzer test [20,21]. The concentration of bovine calmodulin necessary for half maximal activation of phosphorylase kinase (maximal activation 5–7-fold) was 30–70 nM.

uCaM-synthetase was quantitated with the fluphenazine-Sepharose

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affinity adsorption test (FP-test) [9]. The incubation mixture (total volume 100 μl) contained 50 mM Tris-HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 500 μg/ml calmodulin, 50 μg/ml ubiquitin, 0.9–10 mg/ml DEAE-cellulose enriched extract. Unless otherwise stated, the mixtures with calcium contained 1.1 mM $CaCl_2$ and 1 mM EGTA whereas the mixtures without calcium only contained 1 mM EGTA. The specific activity of uCaM-synthetase in the enriched extracts was between 12 and 52 fkat/mg. The heat step which was also employed for other experiments involves the heating of the extract of 100°C for 5 min. The amount of FP-Sepharose employed depended on the amount of calmodulin in the mixture and was held at the ratio of 100 μl packed FP-Sepharose per 100 μg of calmodulin.

Electrophoresis in the presence of SDS was performed on 15% polyacrylamide gels according to [22]. Ca^{2+}-dependent mobility changes of calmodulin [23] during electrophoresis were performed as previously described [8]. The molecular weights were monitored with ovalbumin 45 kDa, glyceraldehyde 3-phosphate dehydrogenase 36 kDa, carbonic anhydratase 29.2 kDa, trypsinogen 25 kDa, trypsin inhibitor 20.1 kDa, lactalbumin 14.2 kDa. For autoradiography the X-ray film was exposed for 40–92 h (see legends) at −80°C and developed for 5–15 min (see legends) as described [9,10]. The autoradiograms were denistometered on a laser scanner (Ultroscan 2202, Pharmacia). For conversion of the peak area to pmol ubiquityl calmodulin conjugate the system was calibrated as described in [8]. Protein [24] was determined on an AutoAnalyzer II (Technicon) employing bovine serum albumin as standard.

3. RESULTS AND DISCUSSION

The ubiquitination of endogenous cellular calmodulin was found more or less by chance when SDS-PAGE-autoradiograms of ubiquitination mixtures, in which ubiquityl-calmodulin had been further purified by affinity adsorption to fluphenazine-Sepharose [9], were overexposed. In a systematic approach the ubiquitination of the unknown protein was followed after the enrichment

Table 1

Amount of endogenous ubiquityl-calmodulin conjugate synthesized in 60 min at 37°C in various enriched tissue extracts

Tissue	Protein (mg/ml)	uCaM-synthetase activity (fkat/mg)	Autoradiographic amount of conjugate formed in 60 min pmol/mg protein exogenous	endogenous	% endog./exog.
Reticulocytes	7.2	52.3	209.1	24.4	12
Erythrocytes	1.8	48.3	181.1	32.0	18
Red muscle	3.3	11.2	48.7	6.8	14
White muscle	2.4	15.0	65.1	12.6	19
Testis	6.2	14.5	48.4	19.1	39

The activity of uCaM synthetase was determined with the FP-test [9]. The net activity given in the table was corrected for the activity found in the absence of exogenous calmodulin (controls). The protein concentration in the incubation mixture was 1/3 the amount given in the table except in the case of reticulocytes where the concentration was 0.9 mg/ml. The net amount of conjugate generated from exogenous or endogenous calmodulin in 60 min at 37°C per mg extract protein was determined by quantitative autoradiography (see Fig. 5 and [8]). The amount of 'autoradiographic' conjugate formed in the presence of exogenous calmodulin has been corrected for the endogenous amount formed in the same time. For further details see Fig. 5, section 2 and the text.

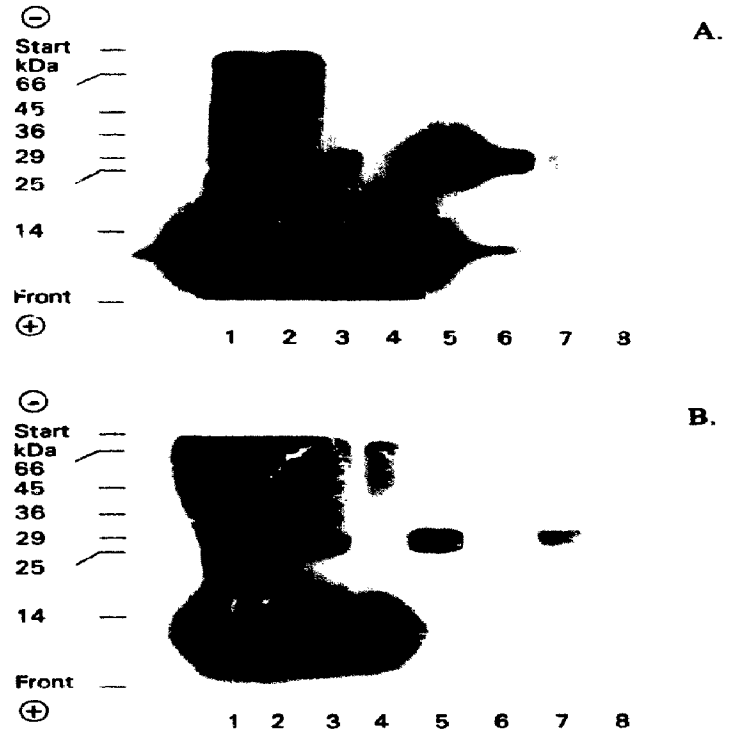

Fig. 1. Ca^{2+}-dependent synthesis of the endogenous ubiquitin conjugate in rabbit testis. The extracts were incubated in mixtures containing as final concentration 50 mM Tris-HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 10 mM phosphocreatine, 0.1 mg/ml creatine kinase, 0.5 mg/ml exogenous calmodulin, 50 μg/ml ^{125}I-CT-ubiquitin (1.4×10^7 cpm/mg) with 0.9 mg/ml DEAE-cellulose enriched reticulocyte lysate or 10 mg/ml DEAE-cellulose enriched extract from testis. Mixtures with calcium (+Ca^{2+}) contained 0.1 mM $CaCl_2$, mixtures without calcium (−Ca^{2+}) contained 1 mM EGTA. The reaction was terminated by boiling after 60 min at 37°C and the precipitated protein was spun down. The supernatant was applied to FP-Sepharose in the presence of Ca^{2+} and conjugates were eluted with EGTA as described in section 2. The EGTA eluates were precipitated in 5% TCA and the pellets were resuspended in sample buffer for 15% polyacrylamide gel electrophoresis [22]. After separation of the polypeptides autoradiograms of the gels (ca. 4 days exposition at −80°C, development for ca. 15 min at room temperature) were prepared. The band running close to the electrophoretic front is unconjugated ^{125}I-ubiquitin. For further details and molecular weight standards see section 2 and the text.

(A)
lane 1: enriched reticulocyte extract endogen. proteins + Ca^{2+}
lane 2: enriched reticulocyte extract endogen. proteins − Ca^{2+}
lane 3: heat treated mixture of lane 2 (supernat.) endogen. proteins + Ca^{2+}
lane 4: heat treated mixture of lane 2 (supernat.) endogen. proteins − Ca^{2+}
lane 5: affinity adsorbed supernatant + exogenous CaM + Ca^{2+}
lane 6: affinity adsorbed supernatant + exogenous CaM − Ca^{2+}
lane 7: affinity adsorbed supernatant endogen. proteins + Ca^{2+}
lane 8: affinity adsorbed supernatant endogen. proteins − Ca^{2+}
(B) Same sample sequence as in (A) except that a mixture containing enriched testis extract was applied to the polyacrylamide gel instead of enriched lysate

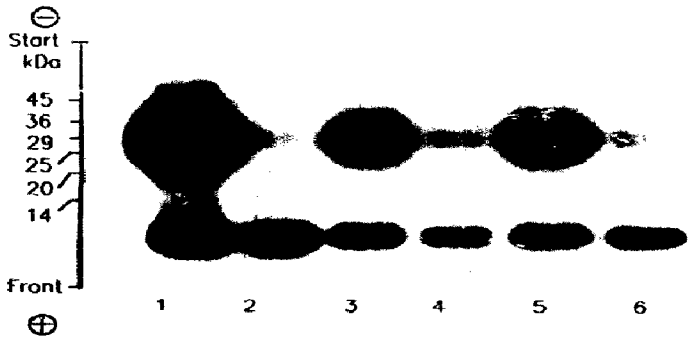

Fig. 2. Autoradiographic detection of an endogenous ubiquitin 29 kDa conjugate in striated red and white rabbit muscle enriched extracts on incubation with ^{125}I-ubiquitin and ATP/Mg^{2+}. The incubation mixture (total volume 0.1 ml) contained as final concentrations 50 mM Tris/HCl, 1 mM DTE, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM $CaCl_2$, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase, 0.5 mg/ml calmodulin, 0.05 mg/ml ^{125}I-ubiquitin (6×10^8 cpm/mg), APF II (reticulocyte 0.9 ml/ml, red muscle 1.1 mg/ml, white muscle 0.8 mg/ml). For further details see legend to Fig. 1, section 2 and the text.

lane 1: reticulocyte APF II + exogenous calmodulin
lane 2: reticulocyte APF II − exogenous calmodulin
lane 3: red (slow twitch) skeletal muscle APF II + exogenous calmodulin
lane 4: red (slow twitch) skeletal muscle APF II − exogenous calmodulin
lane 5: white (fast twitch) skeletal muscle APF II + exogenous calmodulin
lane 6: white (fast twitch) skeletal muscle APF II − exogenous calmodulin

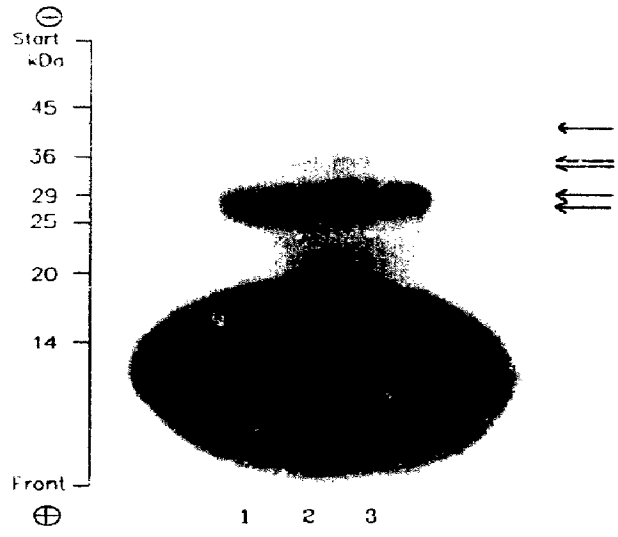

Fig. 3. Ca^{2+}-dependent mobility change of the endogenous ubiquitin conjugate as exemplified in the DEAE fraction of erythrocytes. The incubation mixtures were prepared as described in the legend to Fig. 1. Erythrocyte APF II was added to a final concentration of 0.9 mg/ml. Electrophoresis and autoradiography were performed as described in the legend to Fig. 1 except that the conjugates were not enriched by adsorption to FP-Sepharose: first-order conjugate ca. 29 kDa, second-order conjugate ca. 36 kDa, third-order conjugate ca. 42 kDa. The arrow-pairs indicate the range of the mobility change for the first- and second-order conjugates. In the presence of Ca^{2+} the conjugates run with a lower molecular weight [8]. For further details see section 2, the text and [8].

lane 1: ^{125}I-ubiquitin and ATP/ Mg^{2+} + Ca^{2+} in sample buffer
lane 2: ^{125}I-ubiquitin and ATP/ Mg^{2+} + Ca^{2+} in sample buffer
lane 3: ^{125}I-ubiquitin and ATP/ Mg^{2+} + EGTA in sample buffer

of the extract by a one-step ion-exchange chromatography. This is shown in the presence and absence of Ca^{2+} in the incubation mixture in Fig. 1 for reticulocytes (Fig. 1A) and testis (Fig. 1B) for which a high endogenous content of calmodulin has been reported [25]. Lanes 1 and 2 ($\pm CA^{2+}$) demonstrate that a large number of proteins is ubiquitinated in enriched reticulocyte and testis extracts. The large difference in the film blackening between reticulocytes (Fig. 1A) and testis (Fig. 1B) is due to the fact that 10-fold more protein was applied in the case of testis. Heat treatment of these ubiquitination mixtures eliminates practically all labelled proteins (lanes 3 and 4, $\pm CA^{2+}$) except a band (doublet) running with an identical molecular mass (27–29 kDa) as the conjugate (doublet, [9]) formed from exogenous calmodulin added to the mixture (lanes 5 and 6, $\pm Ca^{2+}$). The calmodulin conjugating activity for exogenous substrate (lane 5) is significantly higher (see also Table I below) in reticulocytes than in testis. The conjugate in lane 6 is due to the Ca^{2+}-independent activity of the enzyme [12]. The adsorption of the supernatant of the heat-treated extract to fluphenazine-Sepharose (lanes 5–8) eliminates free ^{125}I-ubiquitin and other nonspecific conjugates. The detection of conjugate in the molecular weight range of 27–29 kDa in lane 7 clearly illustrates that it is adsorbed and desorbed on FP-Sepharose in a Ca^{2+}-dependent manner and thereby identical to the conjugate observed in lane 3. From the gels it can be concluded that the synthesis of the endogenous ubiquityl conjugate is highly Ca^{2+}-dependent (no conjugates detectable in lanes 4,6,8 $-Ca^{2+}$) and is the major endogenous conjugate remaining in the heat-treated extracts of both reticulocytes and testis.

In Fig. 2 the generation of the same novel endogenous ubiquitin conjugate band in red and white striated skeletal muscle is shown. In lanes 3,5 exogenous calmodulin has been added as control to the incubation mixtures. The endogenous formation of the novel band is shown in lanes 4 and 6 as a faint radioactive band (doublet) visible in the same molecular mass range (27– 29 kDa) as the band formed with exogenous calmodulin (lanes 1,3,5). The conjugating activity is again significantly higher in reticulocytes (lanes 1 and 2) than in striated muscle.

Fig. 3 demonstrates the Ca^{2+}-dependent mobility change (see also [8]) of the novel endogenous conjugate

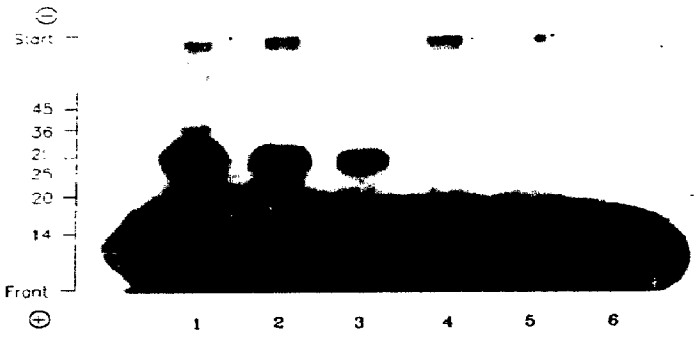

Fig. 4. Inhibition of endogenous conjugate formation in enriched rabbit testis extract by addition of increasing amounts of phosphorylase kinase to the incubation mixture. The incubation mixtures were prepared for the detection of endogenous conjugate as described in the legend to Fig. 1. Testis enriched extract was added to a final concentration of 1.8 mg/ml. The specific radioactivity of ^{125}I-ubiquitin was 1×10^6 cpm/mg. Electrophoresis and autoradiography were performed as described in the legend to Fig. 1 except that the conjugates were not enriched by adsorption to FP-Sepharose. The gels were exposed to the X-ray film for 40 h and developed for 5 min at 5°C. For further details see legend to Fig. 1, Methods, the text and [9].

lane 1: control mixture without phosphorylase kinase
lane 2: mixture + 0.11 mg/ml phosphorylase kinase
lane 3: mixture + 0.46 mg/ml phosphorylase kinase
lane 4: mixture + 1.15 mg/ml phosphorylase kinase
lane 5: mixture + 2.30 mg/ml phosphorylase kinase
lane 6: mixture + 4.46 mg/ml phosphorylase kinase

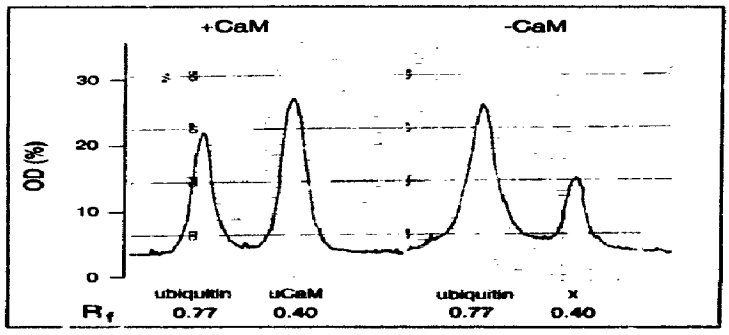

Fig. 5. Quantitative evaluation of the amount of endogenous first-order (27–29 kDa) ubiquitin conjugate in enriched testis extracts by quantitative autoradiography. The incubation mixtures were prepared as described in the legend to Fig. 1. Enriched testis extract was added to a final concentration of 1.8 mg/ml. Electrophoresis and autoradiography were performed as described in the legend to Fig. 1. The autoradiograms were densitometered and evaluated as described in section 2 and [8]. Evaluation of the densitograms yielded 48.4 pmol for net uCaM conjugate from exogenous calmodulin mixture and 19.1 pmol uCaM from endogenous calmodulin alone. $\pm CaM^{2+}$ indicates the presence or absence of *exogenous* calmodulin in the mixture. For further details see Table I, section 2 and the text

+CaM: R_f = 0.4 corresponds to first-order (exogenous) ^{125}I- uCaM
R_f = 0.77 corresponds to free ^{125}I-ubiquitin
−CaM: R_f = 0.4 'X' corresponds to first-order endogenous ^{125}I-uCaM
R_f = 0.77 corresponds to free ^{125}I-ubiquitin

in erythrocyte extract on polyacrylamide gel in the presence of SDS. In addition to the first-order conjugate a second- and third-order conjugate band can be detected. These higher-order conjugates, which have been previously described (see [11]), also exhibit a small Ca^{2+}-dependent mobility change, suggesting that the biological function of calmodulin is still largely intact even after conjugation of 2 or more ubiquitin molecules.

In order to exclude that other Ca^{2+}-binding proteins besides calmodulin might also constitute a conjugate band similar to the one described in Figs. 1–3, a sensitive reaction for the identification of calmodulin under the conditions of conjugation with ubiquitin was applied. Previously it was shown [9] that phosphorylase kinase inhibits the conjugation of calmodulin, probably by competitively binding calmodulin and thereby eliminating it as substrate from the conjugation reaction. Only one other molecule is known to bind phosphorylase kinase under these conditions namely the troponin C subunit of troponin [26], which, however, is not present in non-muscular tissues as testis and which is not present in free form in skeletal muscle [27]. As can be seen in Fig. 4 the amount of endogenous conjugate formed (lane 1) can be titrated to its disappearance (lanes 4–6) by the addition of phosphorylase kinase to the enriched testis extract. This experiment together with the previous ones clearly identifies the novel endogenous conjugate as a calmodulin conjugate of ubiquitin.

Since the previous experiments have allowed the identification of the novel band as ubiquityl calmodulin, its formation in testis extract was evaluated by quantitative scanning [8]. The scans of autoradiograms analogous to the runs in Fig. 2 are shown in Fig. 5. As required, the endogenous conjugate has an identical R_f-value of 0.4 as the conjugate formed from exogenous calmodulin. By this method it can be calculated that on addition of exogenous calmodulin to the incubation mixture from testis (see Table I) a net peak area (corrected for the peak area found in the absence of exogenous CaM, see below) corresponding to ca. 48 pmol uCaM/mg protein and in the absence of exogenous calmodulin a peak area corresponding to ca. 19 pmol uCaM/mg protein is obtained after 60 min of incubation. Thus in enriched testis extract there is enough endogenous calmodulin to generate up to 40% of the amount of conjugate otherwise found only after addition of exogenous calmodulin. In the other tissues (Table I) the amount of endogenously synthesized uCaM (60 min) lies between 7 and 32 pmol/mg protein, i.e. 15–20% of the amount maximally obtained by the addition of exogenous calmodulin.

From the ubiquitous distribution of ubiquitin in all eukaryotic cells [28] it can be concluded that ubiquitin

is initially present in all of the tissue extracts described. Our own measurements (Gehrke and Jennissen, unpublished) have indicated 390 μg/g wet weight of ubiquitin in erythrocytes (see also [29]) and 33 μg/g wet weight in white skeletal muscle (see also [27]). If these values are corrected for cell water content (of e.g. 76% for skeletal muscle [30]), intracellular concentrations of 43–490 μg ubiquitin/ml cell water are obtained. Therefore the addition of ^{125}I-CT-ubiquitin in concentrations of 50 μg/ml to the incubation mixture is in the physiological range. Since it was shown previously that for conjugate formation radiolabelled ^{125}I-CT-ubiquitin is qualitatively indistinguishable from non-derivatized native ubiquitin [11], the conjugation bands are not artifacts of the derivatization procedure for labelling ubiquitin. From the existence of endogenous calmodulin conjugation in these extracts it must be concluded that this reaction is in some way decisive for eukaryotic cell function. Whether this function lies in the regulation of calmodulin degradation or in a specific functional change is still unclear [12].

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